PATENT APPLICATION FEE DETERMINATION RECORD

Effective October 1, 1994

Application or Docket Number: 532211

CLAIMS AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE	SMALL ENTITY FEE	OR	OTHER THAN SMALL ENTITY RATE	OTHER THAN SMALL ENTITY FEE
BASIC FEE				365.00	OR		730.00
TOTAL CLAIMS	24 minus 20 =	* 4	x$11=		OR	x$22=	88
INDEPENDENT CLAIMS	2 minus 3 =	* 0	x38=		OR	x76=	
MULTIPLE DEPENDENT CLAIM PRESENT			+120=		OR	+240=	
			TOTAL		OR	TOTAL	818

* If the difference in column 1 is less than zero, enter "0" in column 2

CLAIMS AS AMENDED - PART II

	(Column 1)		(Column 2)	(Column 3)	SMALL ENTITY RATE	SMALL ENTITY ADDITIONAL FEE	OR	OTHER THAN SMALL ENTITY RATE	OTHER THAN SMALL ENTITY ADDITIONAL FEE
AMENDMENT A	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA					
Total	*	Minus	**	=	x$11=		OR	x$22=	
Independent	*	Minus	***	=	x38=		OR	x76=	
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM					+120=		OR	+240=	
					TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

	(Column 1)		(Column 2)	(Column 3)	RATE	ADDITIONAL FEE	OR	RATE	ADDITIONAL FEE
AMENDMENT B	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA					
Total	*	Minus	**	=	x$11=		OR	x$22=	
Independent	*	Minus	***	=	x38=		OR	x76=	
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM					+120=		OR	+240=	
					TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

	(Column 1)		(Column 2)	(Column 3)	RATE	ADDITIONAL FEE	OR	RATE	ADDITIONAL FEE
AMENDMENT C	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA					
Total	*	Minus	**	=	x$11=		OR	x$22=	
Independent	*	Minus	***	=	x38=		OR	x76=	
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM					+120=		OR	+240=	
					TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20."
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			PCT/		
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PCT/FOREIGN APPLICATION DATA

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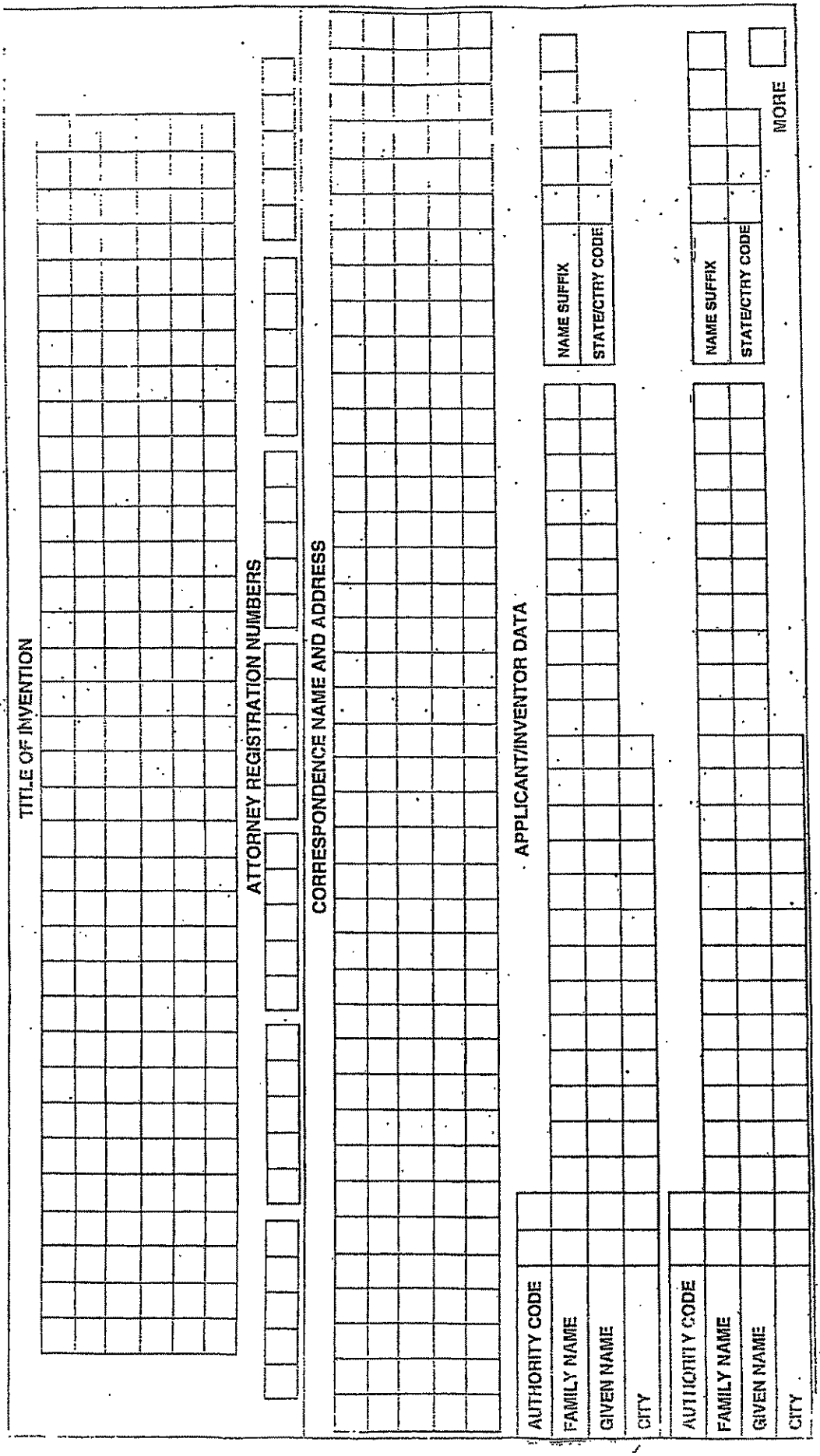
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GIVEN NAME
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STATE/CTRY CODE
AUTHORITY CODE
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CITY
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MORE

Buffers for pH and Metal Ion Control

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London New York
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effectiveness is expressed quantitatively by their *buffer capacity* (see below).

Many organic, and some inorganic, acids and bases have pK_a values between 2 and 12 so that, in principle, by partially neutralizing their solutions they could be used as buffers. Neglecting, for the moment, the effect of ionic strength, solutions of such an acid and its conjugate base in concentration ratios of 1 : 10 to 10 : 1 would furnish a series of buffers covering a pH range of $pK_a \pm 1$.

A solution of a weak acid, or its salt with a strong base, *alone*, is a poor buffer. This is also true of a weak base, or its salt with a mineral acid. In all these cases, the concentration ratio of acid and conjugate base differs markedly from unity so that the addition of strong acid or alkali leads to a rapid initial change in pH. However, the salt of a weak acid and a weak base, for example ammonium acetate, acts as a buffer, alone, because hydrolysis results in measurable amounts of the free acid and the free base in the solution.

2.2 Activity effects

The ionic strength, I, of a solution is given by the summation

$$I = \tfrac{1}{2}\sum(c_i z^2) \qquad (2.9)$$

where c_i is the concentration of each type of ion (in moles l^{-1}) and z is its charge.* Thus, for 0.15 M NaCl, $I = \frac{1}{2}(0.15 \times 1^2 + 0.15 \times 1^2) = 0.15$, and for 0.1M K_2SO_4, $I = \frac{1}{2}(0.2 \times 1^2 + 0.1 \times 2^2) = 0.3$. For solutions outside the pH range 4–10 the contributions of hydrogen and hydroxyl ions must also be included.

The activity of an ion, a_i, is related to its concentration,

$$a_i = c_i f_i \qquad (2.10)$$

*The *molar* scale, moles litre^{-1}, is designated by M to distinguish it from the *molal* scale, m, which is the number of moles dissolved in 1 kg of solvent. The two scales are very similar for dilute aqueous solutions, but are quite different for solutions in mixed solvents.

In dilute solutions, the activity coefficient, f_i, is given by the Debye-Hückel equation, an approximate form of which is (Davies, 1938)

$$-\log f_i = Az^2 I^{\frac{1}{2}}/(1 + I^{\frac{1}{2}}) - 0.1z^2 I \qquad (2.11)$$

where A is a constant which depends on the temperature, being 0.507 at 20°C, 0.512 at 25°C and 0.524 at 38°C.

This equation enables the pH of a buffer solution to be calculated from the thermodynamic pK_a of the buffer acid or base, and the concentrations of the buffer species:

$$pH = pK_a + \log [H_{n-1}A^{(x+1)-}]/[H_nA^{x-}] - (2x+1)AI^{\frac{1}{2}}/(1 + I^{\frac{1}{2}}) + 0.1(2x+1)I \qquad (2.12)$$

$$pH = pK_a + \log [H_nB^{x+}]/[H_{n+1}B^{(x+1)+}] + (2x+1)AI^{\frac{1}{2}}/(1 + I^{\frac{1}{2}}) - 0.1(2x+1)I \qquad (2.13)$$

Example. What is the ionic strength of a pH 7.2 buffer comprising 36 ml 0.2M Na_2HPO_4 and 14 ml 0.2M NaH_2PO_4 in a final volume of 100 ml?

The concentration of HPO_4^{2-} is 0.072M

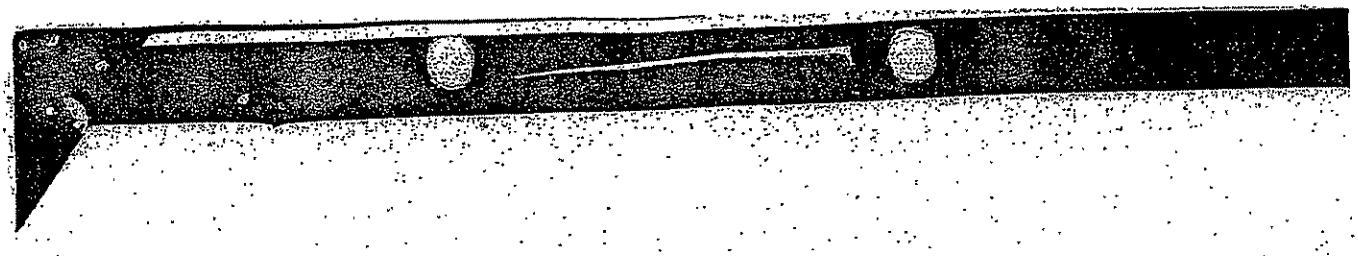
The concentration of $H_2PO_4^-$ is 0.028M

The concentration of Na^+ is $2 \times 0.072 + 0.028 = 0.172$M

$$I = \tfrac{1}{2}(2^2[HPO_4^{2-}] + 1^2[H_2PO_4^-] + 1^2[Na^+])$$
$$= \tfrac{1}{2}(0.288 + 0.028 + 0.172) = 0.244$$

2.3 Effect of dilution

From the relations 2.12 and 2.13, the pH value of a buffer will change with its dilution, because of changes in the ionic strength. Table 2.2 shows the magnitude of the effect of diluting an equimolar solution of a HA/A^- buffer (total molar concentration stated) with an equal volume of water. The quantity $\Delta pH_{\frac{1}{2}}$ is defined as the increase in pH of a solution when it is diluted in this way. Dilution of acidic buffers increases the pH; with bases there is a decrease. Conversely, the addition of an 'inert' salt such as NaCl



THIRD EDITION

QUANTITATIVE ANALYTICAL CHEMISTRY

James S. [redacted]
Iowa State University

George H. Schenk
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ALLYN AND BACON, INC.
BOSTON • LONDON • SYDNEY • TORONTO

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$$\text{Moles of silver } (Ag) = \frac{\text{grams}}{\text{atomic wt}} = \frac{\text{grams}}{107.87}$$

Concentration of solutions. Any one of several different methods can be used to express the concentration of solutions. The systems commonly used are listed in Table 1-1.

In analytical chemistry the molar and normal systems are the most frequently employed. Some chemists use the formal system extensively. Analytical calculations with these systems are discussed in detail later in the book.

It is important to distinguish between the *analytical* concentration and the *equilibrium* concentration of a solution.

TABLE 1-1. *Systems for Expressing Concentrations of Solutions*

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hydronium ion, but is conveniently written H^+ and referred to as the hydrogen ion.

Solutes may be roughly classified according to their ability to ionize and conduct an electric current: nonelectrolytes, such as sugar and urea, weak electrolytes, such as weakly ionized acids and bases, and strong electrolytes, such as HCl or KCl, which are highly or completely ionized in aqueous solution (potassium chloride is known to be completely ionized in the solid state).

Except in very dilute solution the effective concentration of ions in solution (determined by the lowering of the freezing point of water, by electrical conductivity, or by other means) is usually less than the actual concentration of ions known to be present. The term *activity* is used to denote the active or effective concentration of an ion or molecule in solution. Activity may be related to molar concentration through the use of an *activity coefficient*; in the equation

$$a_i = f_i [i]$$

a_i is the activity of an ion, f_i is the activity coefficient of that ion, and $[i]$ is the molar concentration of the ion. In very dilute solution f_i approaches 1; that is, $a_i \approx [i]$. As the concentration of a species increases, the activity coefficient becomes smaller and the values of a_i and $[i]$ become more divergent. The activity coefficient of an ion of charge greater than 1 is smaller at any given concentration than that of an ion whose charge is unity. Activity coefficients of nonionic substances are approximately 1 except in very concentrated solutions.

Differences between concentration and activity arise because of ionic interaction. In a solution of an electrolyte both positively charged and negatively charged ions are moving about. Ions of like charge will repel each other, but ions of unlike charge will attract each other. Attraction and repulsion are not as strong in water as in solvents having lower dielectric constants, but they do exist. Most of the time the space around a positive ion will have an excess negative charge, and the space around a negative ion will, on the average, have an excess positive charge. Thus the motion of the average ion (either plus or minus) is impeded to some extent, and it is not as active as an entirely free ion. As the solution becomes more dilute, the ions in solution are farther apart and have less effect on one another.

From the laws of electrical attraction and repulsion and from the Boltzmann distribution law, which describes the tendency of thermal agitation to counteract electrostatic effects, Debye and Hückel have derived an equation that enables activity coefficients to be calculated theoretically. According to Debye and Hückel, the activity coefficient of any ion depends on the *ionic strength* of the solution. The ionic strength of a solution is *not* equivalent to the total ionic concentration, but is defined by the equation

$$\mu = \tfrac{1}{2} \sum [i] Z_i^2$$

where μ is the ionic strength, $[i]$ is the molar concentration of an ion, and Z_i is the charge (+ or −) of that ion.

Example. Calculate the ionic strength of a 0.1*M* KCl solution. $[K^+] = 0.1$, and $[Cl^-] = 0.1$; then

$$\mu = \tfrac{1}{2}(0.1 + 0.1) = 0.1$$

Example. Calculate the ionic strength of a 0.1*M* Na_2SO_4 solution. $[Na^+] = 0.2$, and $[SO_4^{2-}] = 0.1$; then

$$\mu = \tfrac{1}{2}[0.2 + 0.1(4)] = 0.3$$

In calculations of the ionic strength of a solution, the contribution of weakly ionized substances, such as weak acids, may be ignored.

The Debye-Hückel equation, which relates the activity coefficient of an ion to the ionic strength of the solution, is

$$-\log f_i = 0.5 Z_i^2 \sqrt{\mu}$$

where f_i is the activity coefficient of an ion, Z_i is the charge (+ or −) of that ion, and μ is the ionic strength of the solution. This equation is useful for an estimation of activity coefficients in a solution of any given ionic strength. The activity coefficients in Table 1-2 have been calculated from a form of the Debye-Hückel equation that takes ionic size into account.

TABLE 1-2. *Individual Ion Activity Coefficients as a Function of Ionic Strength*[a]

Ion	Ion size parameter	Activity coefficient				
		$\mu =$ 0.002	$\mu =$ 0.01	$\mu =$ 0.02	$\mu =$ 0.1	$\mu =$ 0.2
H^+	9	0.967	0.933	0.914	0.86	0.83
Li^+	6	0.965	0.929	0.907	0.835	0.80
Na^+, IO_3^-, HSO_4^-	4	0.964	0.927	0.901	0.815	0.77
OH^-, F^-, ClO_4^-	3.5	0.964	0.926	0.900	0.81	0.76
K^+, Cl^-, Br^-, I^-	3	0.964	0.925	0.899	0.805	0.755
NH_4^+, Ag^+	2.5	0.964	0.924	0.898	0.80	0.75
Mg^{2+}, Be^{2+}	8	0.872	0.755	0.69	0.52	0.45
Ca^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}	6	0.870	0.749	0.675	0.485	0.405
Ba^{2+}, Cd^{2+}	5	0.868	0.744	0.67	0.465	0.38
Pb^{2+}	4.5	0.867	0.742	0.665	0.455	0.37
SO_4^{2-}, HPO_4^{2-}	4	0.867	0.740	0.660	0.445	0.355
Al^{3+}, Fe^{3+}, Cr^{3+}	9	0.738	0.54	0.445	0.245	0.18
PO_4^{3-}	4	0.725	0.505	0.395	0.16	0.095
Th^{4+}, Zr^{4+}, Ce^{4+}	11	0.588	0.35	0.255	0.10	0.065

[a] From J. Kielland, *J. Am. Chem. Soc.*, 59, 1675 (1937).

BIOCHEMICAL CALCULATIONS

How to Solve Mathematical Problems in General Biochemistry

Second Edition

IRWIN H. SEGEL
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Davis, California

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$$\text{Ionic Strength } \left(\frac{I}{2}\right) = 1/2 \sum M_i Z_i^2 \qquad (12)$$

where M_i = the molarity of the ion
Z_i = the net charge of the ion (regardless of sign)
Σ = a symbol meaning "the sum of"

Ionic strength measures the concentration of charges in solution. As the ionic strength of a solution increases, the activity coefficient of an ion decreases. The relationship between the ionic strength and the molarity of a solution of ionizable salt depends on the number of ions produced and their net charge, as summarized below.

Salt		Ionic Strength
Type	Example	
1:1	KCl, NaBr	M
2:1	$CaCl_2$, Na_2HPO_4	$3 \times M$
2:2	$MgSO_4$	$4 \times M$
3:1	$FeCl_3$, Na_3PO_4	$6 \times M$
2:3	$Fe_2(SO_4)_3$	$15 \times M$

"Type" refers to the net charge on the ions. Thus $MgSO_4$, which yields Mg^{2+} and SO_4^{2-}, is called a 2:2 salt. Na_2HPO_4, which yields HPO_4^{2-} and Na^+ ions, is called a 2:1 salt.

Only the *net* charge on an ion is used in calculating ionic strength. Thus, un-ionized compounds (e.g., un-ionized acetic acid) or species carrying an equal number of positive and negative charges (e.g., a neutral amino acid) do not contribute toward the ionic strength of a solution.

• Problem 1-3

Calculate the ionic strength of a 0.02 M solution of $Fe_2(SO_4)_3$.

Solution

$$\frac{\Gamma}{2} = \frac{1}{2} \sum M_i Z_i^2 = \frac{1}{2}[M_{Fe^{3+}} Z_{Fe^{3+}}^2 + M_{SO_4^{2-}} Z_{SO_4^{2-}}^2]$$

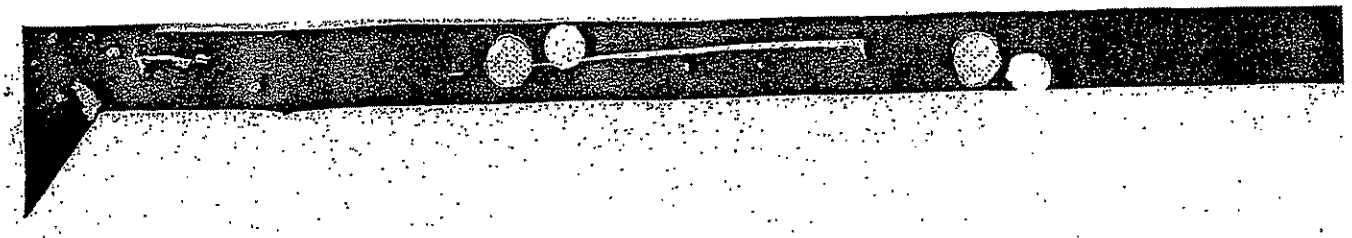
The 0.02 M $Fe_2(SO_4)_3$ yields 0.04 M Fe^{3+} and 0.06 M SO_4^{2-}.

$$\frac{\Gamma}{2} = \frac{(0.04)(3)^2 + (0.06)(-2)^2}{2} = \frac{(0.04)(9) + (0.06)(4)}{2}$$

$$= \frac{(0.36) + (0.24)}{2} = \frac{0.60}{2}$$

$$\frac{\Gamma}{2} = 0.30$$

NO UNITS



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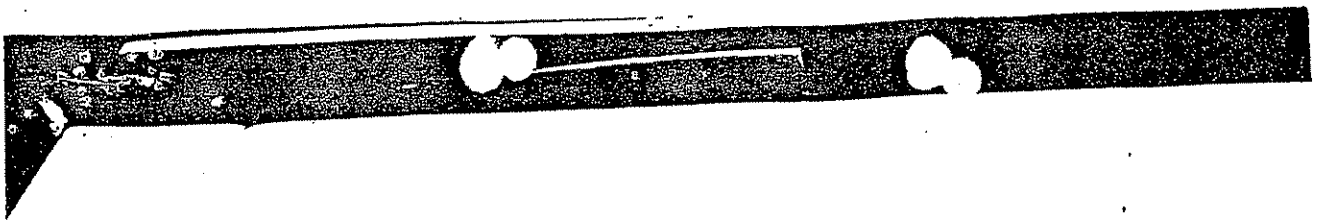

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Clinical Immunology and Immunopathology
Volume 34, Number 2, February 1985

CONTENTS

CLINICAL IMMUNOLOGY AND IMMUNOPATHOLOGY 34, 227–236 (1985)

Intravenous and Standard Immune Serum Globulin Preparations Interfere with Uptake of ^{125}I-C3 onto Sensitized Erythrocytes and Inhibit Hemolytic Complement Activity[1]

MELVIN BERGER,[2] PNINA ROSENKRANZ,[3] AND CARMEN Y. BROWN

Allergy–Immunology Service, Walter Reed Army Medical Center, Washington, D.C. 20307

Antibody-sensitized sheep erythrocytes were used as a model to determine the effects of therapeutic immune serum globulin (ISG) preparations on the ability of this particulate activator to fix C3 and initiate hemolysis. Both standard and intravenous forms of ISG inhibit uptake of ^{125}I-C3, presumably by competing for the deposition of "nascent" C3b molecules onto the erythrocytes. Both forms of ISG also inhibit hemolytic activity of whole serum or purified complement components. The inhibition appears to be a specific property of IgG itself, since similar inhibition was not caused by equivalent concentrations of human serum albumin, and was not affected by the buffer in which the ISG was dissolved. Interference with C3 uptake onto antibody-sensitized platelets and/or inhibition of hemolytic complement activity could contribute to the efficacy of high dose intravenous ISG in idiopathic thrombocytopenic purpura.

INTRODUCTION

Although it has only been three years since Imbach *et al.* (1) first reported the successful treatment of idiopathic thrombocytopenic purpura (ITP)[4] with high doses of intravenous immunoglobulin (IVIG), this form of therapy has come into widespread use. Despite a number of studies, however, the means by which high levels of IgG cause prolonged survival of sensitized platelets remains unknown (2, 3). Blockade of the Fc receptors in the reticuloendothelial system (RES) has been postulated to be a primary site of action of IVIG and prolongation of the clearance rate of sensitized erythrocytes has been reported (4–6). The mechanism by which monomeric IgG could inhibit phagocytosis of multivalent sensitized particles has not been clearly elucidated, however, and other possible explanations for the efficacy of IVIG are the subject of continuing investigation (2–6).

There are two primary routes by which the serum complement system can

[1] Supported by the Department of Clinical Investigation, Walter Reed Army Medical Center. The opinions and assertions contained herein are the privately held views of the authors and are not to be construed as official policies of the Department of the Army or of the Defense Department.

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[4] Abbreviations: ISG, immune serum globulin; IVIG, immune serum globulin for intravenous infusion; E, sheep erythrocyte; EA, sheep erythrocyte sensitized with rabbit anti-forssman antibody; EAC14, EA bearing the first and fourth components of complement; ITP, idiopathic thrombocytopenic purpura; HSA, human serum albumin; RES, reticuloendothelial system; C3, the third component of complement, and C3b, its major cleavage fragment; PBS, phosphate-buffered saline, pH 7.4.

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participate in autoimmune destruction of formed elements of the blood that have become sensitized with antibody. Deposition of C3b onto particles allows them to interact with C3b receptors on RES macrophages and can enhance Fc mediated phagocytosis, while continued activation of the complement system beyond C3 may lead to formation of the membrane attack complex and cause intravascular lysis. The possibility of participation of these complement pathways in the destruction of platelets in ITP is suggested by observations that freshly obtained platelets from the circulation of patients with ITP bear increased amounts of C3 on their surface (7, 8) and that antibodies from ITP patients cause C3 fixation onto platelets *in vitro* (9, 10). In addition, recent studies have demonstrated that anti-platelet antibodies can activate complement by the classical pathway and lead to deposition of terminal components including C9 onto the platelet membranes (11).

When C3 is activated an internal thiolester becomes transiently exposed, enabling the "nascent" C3b to bind covalently to suitable acceptors with –OH or $-NH_2$ groups by a transacylation reaction (12, 13). A variety of recent studies suggest that IgG is a particularly good acceptor for C3b during complement activation by soluble immune complexes (14) as well as by bacteria (15), and it had previously been observed that addition of fluid phase IgG caused diminished binding of C3 to particles after *in vitro* activation by trypsin (16).

In the present studies we used antibody-sensitized sheep erythrocytes as a model system for classical pathway activation and attempted to determine whether the addition of excess fluid phase IgG would interfere with C3 deposition onto these particles and/or reduce their lysis by complement. The results clearly demonstrate that therapeutic preparations of IgG inhibit deposition of ^{125}I-C3b onto the sensitized erythrocytes and diminish the hemolytic activity of serum or purified complement components. Similar inhibition of complement function *in vivo* could contribute to the therapeutic efficacy of high dose IVIG infusions in ITP.

MATERIALS AND METHODS

Immunoglobulin and albumin preparations. Standard preparations of human immune serum globulin, USP (ISG) and 25% human serum albumin (HSA) were obtained from the hospital pharmacy. Immune serum globulin, 5% in 10% maltose for intravenous infusion (IVIG) was obtained from Cutter Biologicals as standard commercial lots of Gamimune stocked by the hospital pharmacy. Protein concentrations in dilutions of these materials were determined by the absorbance at 280 nm using the extinction coefficients ($E^{1\%}_{1\,cm}$) 5.3 for HSA and 14.3 for immunoglobulins (17).

Complement components and cellular intermediates. Functionally pure human complement components C1, C4, C2, C5, C6, and C7 and guinea pig C8 and C9 were purchased from Cordis Laboratories, Miami, Florida. Human C3 was purified from pooled plasma as previously described (18, 19) and retained the native specific hemolytic activity of C3 in unfractionated serum as determined by hemolytic assay (19, 20). C3 was labelled with ^{125}I using "Iodobeads" (Pierce Chemical Co., Rockford, Ill.) and carrier free Na^{125}I (Amersham-Searle, Chicago, Ill.)

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to a specific radioactivity of 1 μCi/ug. Immediately before use in uptake studies ^{125}I-C3 preparations were centrifuged at 100,000*g* for 10 min in a Beckman Airfuge to ensure the absence of aggregates.

Rabbit anti-Forssman antibodies were the kind gift of Thelma Gaither, NIH, Bethesda, Maryland. Isotonic veronal buffered saline with 0.1% gelatin and isotonic dextrose–veronal buffered saline with gelatin were prepared as previously described (21). Sheep erythrocytes (E) were sensitized with antibody and in some cases, C1 and C4 as previously described (19, 21).

Serum was prepared by allowing freshly drawn blood from normal fasting donors to clot at room temperature for 45 min and then on ice for an additional 1 hr, followed by centrifugation at 4°C. Serum from a patient genetically deficient in C6 who was otherwise in good health was handled similarly. All serum samples were stored at −70°C until use.

Hemolytic assays. E sensitized with antibody alone (EA) or with antibody and C1 and C4 (EAC$\overline{14}$) were incubated for 1 hr at 37°C with a mixture of the protein to be tested for inhibitory activity and/or the appropriate buffer for that protein preparation, as well as a dilution of whole serum or a mixture of a limiting amount of purified C3 (1–2 units) and a developing reagent consisting of excess (100 units/ml) purified C2 and C5-9. In most cases, 0.1 ml of EA or EAC $\overline{14}$ at 1.5 × 10^8 cells/ml was used in a total reaction volume of 0.5 ml. Following incubation, 2 ml of ice-cold phosphate buffered saline was added to each tube, the remaining intact cells were pelleted by centrifugation at 2500*g* for 5 min at 4°C and the degree of hemolysis was determined by measuring the absorbance at 412 nm of the hemoglobin in the supernatant. In all experiments, blanks without serum or developing reagent, but including cells and inhibitor proteins, as well as tubes with serum and inhibitor protein but no cells were run concurrently. The total amount of hemoglobin present was determined in tubes containing distilled water rather than the other reaction constituents. In the experiments measuring hemolysis by whole serum, results are expressed as percentage lysis in experimental tubes compared to appropriate controls. In experiments measuring hemolysis by purified C3 and developing reagent, results are expressed as C3 hemolytic sites per cell determined as described previously (20). In all cases, results shown are the average of duplicate determinations.

^{125}I-C3 uptake. Reaction tubes contained 0.025 ml EA or EAC $\overline{14}$ at 3 × 10^8/ml, 0.025 ml of purified C2 or diluted serum from an individual genetically deficient in C6 but otherwise well at the time serum was obtained, 0.10 ml ^{125}I-C3 and 0.10 ml of a mixture of inhibitor protein to be tested and appropriate buffer. The input of ^{125}I-C3 was verified by counting each tube in a γ scintillation spectrometer before starting the reaction with C2 or serum. All tubes were incubated 1 hr at 37°C then duplicate 100-μl aliquots were withdrawn and layered over 250 μl of 25% albumin in 400-μl polypropylene centrifuge tubes. Tubes were spun 5 min at 10,000*g* in a microfuge, and the tips containing the pelleted erythrocytes were cut off and counted to determine the amount of ^{125}I-C3 bound. Blanks with all reactants except C2 or C6 deficient serum were run in parallel and the counts bound in these tubes were considered background and subtracted from all experimental tubes. Results of the duplicate aliquots from each tube were averaged

and corrected for the total reaction volume by multiplying by 2.5. All experimental tubes were run in duplicate with the results shown representing the average ^{125}I-C3 binding compared to the appropriate controls.

RESULTS

Inhibition of hemolytic activity. Based on the results of preliminary experiments, we selected dilutions of normal serum that would give appropriate amounts of lysis of sensitized erythrocytes and then determined the effect of adding various commercial preparations of human serum immune globulin on the degree of hemolysis observed. We also included human serum albumin as a control. For each protein preparation, an equivalent buffer control was also run concurrently. As can be seen in Fig 1, standard immune serum globulin for intramuscular injection (ISG) was very inhibitory even at a final concentration of 1 mg/ml. The intravenous immunoglobulin (IVIG) preparation also caused marked inhibition but required 17.5 mg/ml for 50% reduction of hemolysis. These effects are likely to be due to the immune globulin in the preparations per se, since the data have been normalized for comparison to the appropriate buffer for each commercial preparation, and human serum albumin caused no inhibition.

Hemolysis of EA is a complex process that may be inhibited at more than one stage. We therefore attempted to define further the site of inhibition by the immunoglobulin preparations by testing their ability to interfere with lysis of the preformed intermediate EAC $\overline{14}$ by limiting amounts of purified C3 in the presence of excess amounts of the remaining components, C2 and C5 through C9. As shown in Fig. 2, ISG and IVIG were markedly inhibitory with 50% reduction in the formation of C3 hemolytic sites requiring 5–6 mg/ml of either preparation.

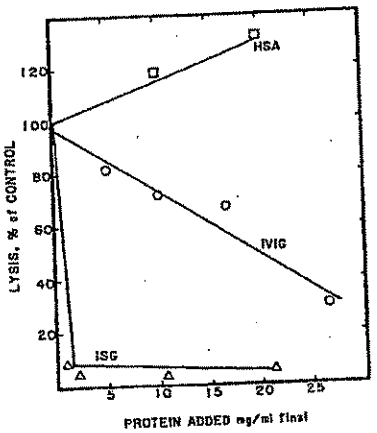
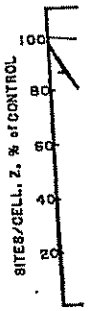


FIG. 1. Hemolysis of EA by whole serum. Serum was used at 1:120 or 1:160 dilution. The control buffer for ISG was 0.3 *M* glycine, pH 6.8 which gave 79% hemolysis. The control for the IVIG was 10% maltose, 0.1 *M* glycine, pH 6.8 which gave 55% hemolysis and the control for HSA was normal saline, pH 7.0 which gave 22% hemolysis.

ISG INHIBITS



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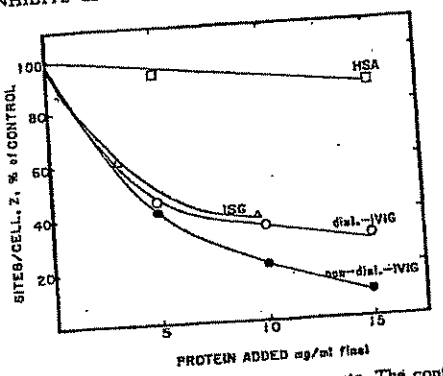

FIG. 2. Hemolysis of EAC $\overline{14}$ by C3 with purified components. The control for ISG was 0.3 *M* glycine, pH 6.8 which resulted in $Z = 1.60$. PBS, pH 7.4 was used as a control for dialyzed (dial.) IVIG and gave $Z = 0.324$. 10% maltose, 0.1 *M* glycine, pH 6.8 was the control for non-dial-IVIG and gave $Z = 1.12$; and normal saline, pH 7.0 was the control for HSA and gave $Z = 1.06$.

To rule out pH or buffer effects we also tested IVIG that had been dialyzed against PBS at pH 7.4. The dialyzed material caused inhibition that was comparable to the nondialyzed IVIG which is maintained in 10% maltose–0.1 *M* glycine pH 6.8, even though the overall hemolytic system was much less active in PBS. Once again, HSA was not inhibitory. The fact that in this assay, IVIG and ISG gave equivalent inhibition suggests the possibility that in the previous experiment, the increased inhibitory activity of standard ISG may be partly due to inhibition of C1 activation.

Inhibition of ^{125}I-C3 uptake. The observation that generation of C3 hemolytic sites on EAC $\overline{14}$ was inhibited by the immune serum globulin preparations suggested that the immunoglobulin might actually be competing with the surface for deposition of the "nascent" C3b molecules. To test this hypothesis, we employed highly purified ^{125}I-C3 so that the uptake of this component could be measured directly. As shown in Fig. 3, both IVIG and ISG inhibited the uptake of ^{125}I-C3 onto EA. In this experiment, C6-deficient serum was used as a source of early components so that lysis of the erythrocytes would not be possible. As in the hemolytic assays, HSA was not inhibitory. To rule out effects of the immunoglobulin preparations on the early activation steps of the complement cascade, we once again employed preformed EAC $\overline{14}$. With C6 deficient serum as a source of C2, marked inhibition was caused by ISG and IVIG but not by HSA, as shown in Fig. 4. Similar results were obtained with purified C2 itself, as shown in Fig. 5. It is unlikely that these effects result from the maltose or glycine in the protein preparations since comparable results were obtained with dialyzed IVIG in PBS and the nondialyzed IVIG in maltose–glycine. These results strongly suggest that the added immunoglobulin directly interferes with C3b binding after activation by the preformed convertase.

Evidence that monomeric IgG is inhibitory. Since earlier studies had indicated that standard ISG preparations contained aggregates that had anticomplimentary

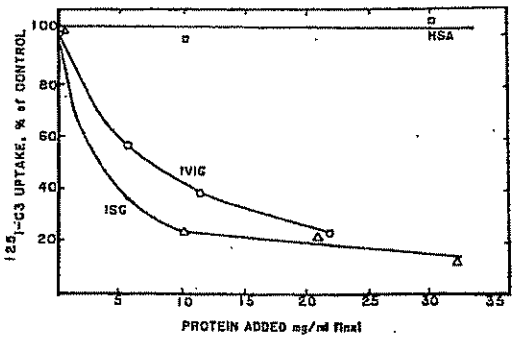

FIG. 3. Uptake of ^{125}I-C3 onto EA with C6 deficient serum. The final serum concentration was 2.5% and the input of ^{125}I-C3 was 1.5×10^6 cpm (1.5 μg)/tube. Control buffers were as in Fig. 2, and the specific uptake in the control tubes was 24,000–35,1000 cpm.

activity, we wished to determine whether the inhibition we observed in these studies was due to the presence of aggregates in the IVIG preparation. A 3-ml sample of IVIG was subjected to gel filtration on a calibrated column of Bio-Gel A5m (Biorad, Inc. Richmond, Calif.) and the fractions were tested for their ability to inhibit the hemolytic activity of whole serum. As can be seen from the elution profile in Fig. 6, there were no large aggregates in this IVIG preparation but the peak is somewhat asymmetric suggesting that there may be small amounts of dimers or oligomers. Inhibitory activity was found in fractions 80–85 which correspond to the elution position of isolated, monomeric IgG. In other experiments, we found that centrifugation of the IVIG at 110,000*g* for 30 min had no effect on its ability to inhibit hemolytic activity (data not shown), again suggesting that aggregates are not present and are not responsible for the observed effects.

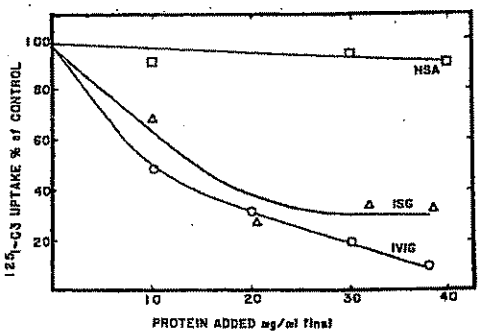

FIG. 4. Uptake of ^{125}I-C3 onto EAC $\overline{14}$ with C6 deficient serum. Serum and ^{125}I-C3 concentrations were the same as in Fig. 3. Control buffers were as in Fig. 2, and the specific uptake in the control tubes was 26,300–35,800 cpm.

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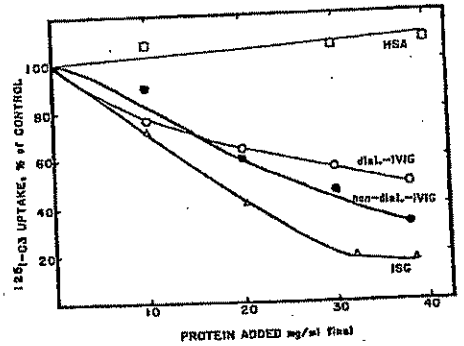

FIG. 5. Uptake of ^{125}I-C3 onto EAC $\overline{14}$ with C2. The input of ^{125}I-C3 was 1.6×10^6 cpm/tube. Control buffers were as in Fig. 2 and the specific uptake in the control tubes was 75,850–84,700 cpm.

DISCUSSION

In these studies we used antibody-sensitized sheep erythrocytes as a model to determine the effects of adding therapeutic preparations of immune serum globulin on the ability of this particulate activator to fix C3 and to initiate hemolysis. The results clearly demonstrate that both standard and intravenous forms of human immunoglobulin inhibit generation of hemolytic sites and interfere with binding of C3 to the particles. This inhibition appears to be a property of the IgG itself since similar inhibition was not caused by human serum albumin at equivalent concentrations and was not dependent on the buffer in which the immunoglobulin was dissolved.

In the past few years there has been a major advance in our understanding of the biochemistry of C3 activation with the demonstration that the intact C3 molecule contains an internal thiolester (12, 22). Following cleavage of the α chain

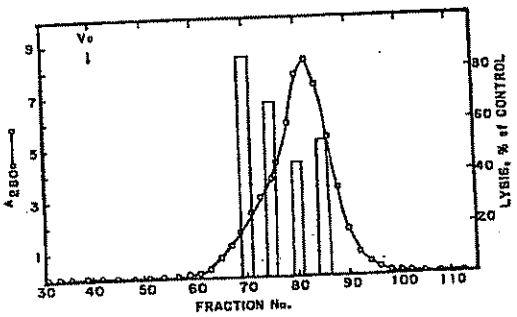

FIG. 6. Inhibition of hemolysis of EA by gel filtration fractions of IVIG. A 1.5 × 100-cm column was used and 1.5-ml fractions were collected. The void volume (Vo) was at fraction 39, as indicated, and chemically purified monomeric IgG eluted with a peak in fraction 84. The final protein concentration of each fraction tested was 1.2 mg/ml. A 1:160 dilution of normal serum was used which gave 22% lysis in the control tube with PBS alone.

of C3, this reactive group is exposed and can participate in a transacylation reaction yielding C3b covalently bound to an acyl acceptor on the activator (13). A series of studies by Law *et al.* first demonstrated that most of the C3b deposited on sheep erythrocytes was bound by ester linkages, presumably to surface carbohydrates (23, 24). More recently, it has been demonstrated that when complement is activated by soluble antigen–antibody complexes, C3b can become bound to antibody molecules by amide as well as ester linkages (14). Brown *et al.* have shown that classical pathway activation by anti-pneumoccal antibody also results in the formation of covalent C3b–antibody complexes some of which are amide linked (15). If a suitable acceptor is not readily available for reaction with the "nascent" C3b, the solvent water will hydrolyze the exposed thiolester and formation of fluid-phase C3b will result. Law *et al.* demonstrated that small molecules such as sugars and amino acids could preferentially compete with solvent water for reaction with fluid phase "nascent" C3b activated by trypsin (25). Similarly, Capel *et al.* had previously shown that a variety of substances including complex carbohydrates and immunoglobulin molecules could compete for binding of C3 activated by trypsin in the fluid phase and inhibit C3b deposition onto a particulate acceptor (16). In these studies we have demonstrated that excess fluid phase immunoglobulin can compete for C3 specifically activated by a particle bound classical pathway convertase. This observation extends the previous chemical studies which used the nonspecific activator trypsin, to a potentially physiologically relevant situation such as that found in autoimmune cytopenia. As we were initially motivated to carry out these studies by the reports of successful treatment of ITP with high dose IVIG, we conclude that interference with C3 deposition and hemolytic complement activity may contribute to the efficacy of this form of treatment. Competition by excessive levels of nonimmune IgG may also be responsible for the diminished binding of C3 to pneumococci recently reported in sera from patients with multiple myeloma (26).

Most previous studies on the interaction of therapeutic immunoglobulin preparations with the serum complement system have focused on direct activation of complement as an explanation for side effects of intravenous infusions, or with the ability of modified immunoglobulin molecules to activate the complement cascade (27–29). In those experiments serum has usually been incubated with the immunoglobulin preparation in its native or heat aggregated state for a fixed period of time then the remaining hemolytic activity or consumption of C3 is determined (28, 29). Our approach differs in that we have examined the effects of the immunoglobulin preparations on activation induced by an independent activating particle, EA or EAC $\overline{14}$. In the case of EA it is possible that some of the inhibition we observed is due to the interaction of C1 with the added immunoglobulin rather than the surface bound anti-erythrocyte antibody. This is suggested by the known interaction of fluid phase monomeric IgG with C1 (30, 31) and it has been demonstrated directly that excess monomeric IgG can inhibit complement-mediated lysis of tumor cells (32). It has also been shown that therapeutic ISG preparations can interfere with binding of ^{125}I-labeled C1q to IgG immobilized on plastic surfaces (29). Inhibition of C1 binding or activation is unlikely to account for the inhibition of hemolytic activity toward EAC $\overline{14}$ or for

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interference with C3 activation by the convertase formed when excess C2 is added to the preformed $\overline{14}$ enzyme, since the EAC4 site is known to have a higher affinity for C1 than fluid phase complexes of EA alone (33) which is the basis for its use in the C1 fixation and transfer test and in assays of C1 activity. We observed that a great difference in concentration of IVIG relative to standard ISG was required to inhibit hemolysis of EA (Fig. 1). This also suggests that the inhibition of EA hemolysis might be dependent on interference with C1 activation, as Bing has demonstrated that the Cutter IVIG preparation, which has been reduced and alkylated, has a lower affinity for C1 than does the standard ISG (29). In contrast, however, the assays of C3 hemolytic activity and uptake which employ EAC $\overline{14}$ showed comparable degrees of inhibition at similar concentrations of IVIG and ISG, implying that a different mechanism of inhibition was involved. We believe that the mechanism of inhibition of C3 uptake by both of these therapeutic immunoglobulin preparations involves direct competition by fluid phase IgG for the nascent C3b with probable formation of IgG–C3b complexes. This mechanism may operate *in vivo* at the extremely high IgG concentrations achievable with intravenous therapy, and could contribute to the efficacy of IVIG in ITP.

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